

REMARKS/ARGUMENTS

With this amendment, claims 1-8, 16, and 28-29 are pending. For convenience, the Examiner's rejections are addressed in the order presented in the October 18, 2004 Office Action.

I. Status of the claims

Claim 1 is amended to correct antecedent basis of the term "malignant cell." This amendment is not a limiting amendment and adds no new matter.

II. Rejections under 35 U.S.C. §112, second paragraph

Claim 1 is rejected as allegedly indefinite for insufficient antecedent basis of the term "malignant cell." Applicants have corrected the use of "a" and "the" in relation to the term "malignant cell" within the claim. In view of this amendment, withdrawal of the rejection under 35 U.S.C. §112, second is respectfully requested.

III. Rejections under 35 U.S.C. §112, first paragraph, written description

Claims 24 and 26-30 are rejected under 35 U.S.C. §112, first paragraph for allegedly failing to comply with the written description requirement. According to the Office Action, the specification lacks description of the claimed invention, such that a skilled artisan would recognize that Applicants had possession of the claimed invention at the time of filing.

The Office Action indicates that the rejection is a new matter rejection and is made because of addition of the phrase "the antibody inhibits growth of a malignant cell that expressed the frizzled 5 receptor" in claims 1 and 16 and because of addition of the phrase "wherein the antibody is effective for immunotherapy of a malignant cell that overexpresses the frizzled 5 receptor" in claim 28. According to the Office Action, the specification does not provide support for the amendment. Applicants respectfully traverse and assert that the specification provides sufficient support for the claimed antibodies. Moreover, according to the

MPEP, there is no *in haec verba* requirement for newly added claim limitations and those limitations can be supported by express, implicit, or inherent disclosure. MPEP 2163 IB.

The application discloses antibodies that bind to extracellular epitopes of frizzled proteins. Frizzled 5 is a member of the frizzled protein family disclosed throughout the specification, *e.g.*, at page 8, lines 3-5 and at Table I, page 9. At page 28, lines 9-12, the specification discloses that, although frizzled 2 is disclosed as an exemplary frizzled protein for the development of antibodies that inhibit malignant cell growth or that can be used as immunotherapies, other frizzled proteins, *e.g.*, frizzled 5, can be used as antigens to develop antibodies for use in the disclosed methods.

With regard to the rejection of claims 1 and 16 for recitation of the phrase "the antibody inhibits growth of a malignant cell that expressed the frizzled 5 receptor," the specification at page 22, lines 14-16 discloses that the anti-frizzled antibodies affect cellular function by, *e.g.*, cell growth inhibition. Thus, this cited claim amendment does not add new matter.

With regard to the rejection of claim 28 for recitation of the phrase "wherein the antibody is effective for immunotherapy of a malignant cell that overexpresses the frizzled 5 receptor," at page 21, line 24 through page 22, line 2 discloses use of antibodies that bind to the extracellular portion of a frizzled protein, *e.g.*, frizzled 5 antibodies, and that kill or inhibit growth of tumor cells on binding, and so can be used for immunotherapy. In addition, immunotherapy using anti-Fz antibodies is also described at page 26, lines 6-13. Thus, this cited claim amendment does not add new matter.

Applicants also point out that the Federal Circuit has ruled that "as long as an applicant has disclosed a 'fully characterized antigen,' either by its structure, formula, chemical name, or physical properties, or by depositing the protein in a public depository, the applicant can then claim an antibody by its binding affinity to that described antigen." *Noelle v. Lederman*, 69 USPQ2d 1508, 1514 (Fed. Cir. 2004). The structure of the Fzd 5 antigen is disclosed as SEQ ID NO:68 in the application.

In view of the above remarks, withdrawal of the rejection for alleged lack of written description is respectfully requested.

IV. Rejections under 35 U.S.C. §112, first paragraph, enablement

Claims 1-8, 16, 28, and 29 are rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement. According to the Office Action, the specification does not enable one of skill to make and/or use the invention commensurate in scope with the claims. The Office Action also alleges that undue experimentation is required to practice the claimed invention. To the extent the rejection applies to the amended claims, Applicants respectfully traverse the rejection.

The Office Action alleges that the specification does not teach how to make or use the claimed frizzled 5 antibodies, and that such technology is unpredictable and would require undue experimentation on the part of those of skill. First, Applicants submit that the disclosure adequately teaches how to make and use the claimed antibodies and how to identify malignant cells that overexpress a frizzled 5 antigen. Second, even if the claimed frizzled 5 antibodies are not therapeutic for treatment of all cancers, claims reading on inoperative embodiments are enabled if the skilled artisan understands how to avoid inoperative embodiments. (*See, In re Cook and Merigold*, 169 USPQ 299, 301 (C.C.P.A. 1971)). In the present application, one of skill would know how to avoid inoperative embodiments and identify the claimed antibodies without undue experimentation. Moreover, the present application provides guidance in the form of assays and working examples for inhibition of malignant cell growth using anti-frizzled antibodies.

The specification teaches that frizzled proteins are overexpressed in certain cancers and uses the frizzled 2 protein as an example for the frizzled protein family. *See, e.g.*, specification, Examples 1 and 2, pages 23-26. The specification also discloses that the wnt/fzd pathway is implicated in tumorigenesis and that this signaling pathway converges on regulation of the β -catenin protein, a transcription factor that is linked to carcinogenesis. *See, e.g.*, specification at page 7, lines 16-26. The specification teaches that blockage of the wnt/fzd pathway signaling can limit tumor cell growth, that inhibition of frizzled signaling can induce apoptosis, and that anti-frizzled antibodies that block signaling can be used to limit tumor cell growth by immunotherapy. *See, e.g.*, specification at page 21, line 24 through page 22, line 9

and page 26, line 6 through page 28, line 2. Again, the frizzled 2 protein is used as an example for the frizzled protein family and the specification teaches that for some cancers other frizzled proteins, including frizzled 5, can be used. *See, e.g.*, specification at page 28, lines 6-12.

The specification teaches how to make and use the claimed antibodies. Antibody production is disclosed at pages 18-20 of the specification. Applicants also submit as Exhibit A, a printout of an Antibody Resource website listing 89 suppliers of custom antibodies. The website is dated April 5, 2001 and was obtained from a website archive, the Wayback Machine, www.archive.org. The earliest priority application was filed on May 1, 2001. Thus, the technology of antibody production was well-known to and commercially available to those of skill in the art at the time of filing.

Methods to detect overexpression of Frizzled 5 gene products using nucleic acid hybridization and amplification techniques, including specific primers, are disclosed at pages 18-20 of the specification. Methods to detect overexpression of Frizzled 5 protein using specific antibodies are disclosed at pages 20-21 of the specification. The Office Action alleges that undue experimentation would be required to identify cancers that overexpress frizzled 5. However, the techniques above could be routinely carried out by laboratory technicians allowing routine screening of large numbers of cells if necessary. Moreover, panels of cancer cells and normal cells for expression screening were available to the user at the time of filing. *See, e.g.*, specification at page 28, lines 13-20. The disclosed panel of cancer cells includes, *e.g.*, a renal cancer cell line.

The Office Action also appears to allege that frizzled 5 is not overexpressed in cancer cells as taught by the specification. To support the specification's disclosure of frizzled 5 overexpression in cancer cells, Applicants submit as Exhibit B Janssens *et al.*, Tumor Biology 25:161-171 (2004); which discloses that the frizzled 5 protein is overexpressed in renal cell carcinomas. The Frizzled 5 overexpression was detected using nucleic acid hybridization and amplification assays and by detection of the Frizzled 5 protein using antibodies. Thus, the submitted reference confirms the disclosure of frizzled 5 overexpression found in the specification. Janssens *et al.* cite a paper by Zang *et al.*, which is submitted herein in an IDS.

Applicants respectfully assert that Zang *et al.* does not disclose the claimed antibodies directed against SEQ ID NO:68.

The Office Action appears to allege that pharmaceutical use of the claimed antibodies must be demonstrated to fulfill the enablement requirement. This is incorrect. The enablement requirement can be met by using an in vitro or in vivo model that correlates with a given condition. Applicants have provided an in vitro system of testing administration of the claimed antibodies for inhibition of cancer cell growth or apoptosis of cancer cells. See, *e.g.*, specification, Examples 3 and 4, at pages 26-28. Applicants remind the Examiner that a rigorous or exact correlation is not required. MPEP 2164.02. One of skill would recognize that this model correlates with the pharmaceutical uses of the claimed antibodies.

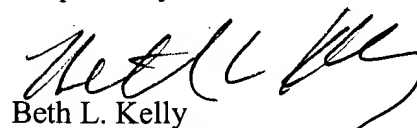
In view of the above remarks and evidence, withdrawal of the rejection for alleged lack of enablement is respectfully requested.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

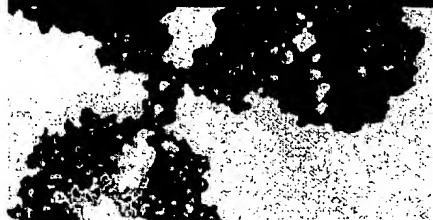
If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,


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Custom Monoclonal and Polyclonal Antibody Suppliers:

Here is a list of custom antibody suppliers with a brief categorization of the kind of services that they provide: Note: The descriptor "peptides" indicates that company will synthesize peptide haptens for you.

If you are interested in general antibody suppliers, see our [online companies](#) page. If you are interested in the large scale, regulatory compliant production of antibodies, see our [contract antibody suppliers](#) page.

If you would like to have your company added to the list or update/correct any information, please click [here](#).

1. [AbCam Ltd](#) - (monoclonal, polyclonal)
2. [NEW AbGenomics](#) - (monoclonal, polyclonal) - antibodies from cDNA
3. [Advanced ChemTech](#) - (polyclonal, peptides)
4. [Affiniti Research Products Ltd.](#) - (UK) - (polyclonal, peptides)
5. [Affinity BioReagents, Inc.](#) - (polyclonal)
6. [AgriSera AB](#) - (monoclonal, polyclonal, peptides)
7. [Alpha Diagnostics](#) - (monoclonal, polyclonal, peptides)
8. [Anaspec](#) - (polyclonal, peptides)
9. [Anawa Trading Company SA](#) - (monoclonal, polyclonal, peptides)
10. [Antibody Solutions](#) - (monoclonal, polyclonal, peptides) - *in vitro* production
11. [Antibodies Incorporated](#) - (monoclonal, polyclonal)
12. [Aurora Biomolecules](#) - (polyclonal, peptides)
13. [Aves Lab](#) - (polyclonal) - chicken antibodies
14. [B & K Universal, Ltd.](#) - (monoclonal, peptides)
15. [Babraham Technix](#) - (monoclonal, polyclonal)
16. [Berkeley Antibody Company](#) - (monoclonal, polyclonal)
17. [Bethyl Laboratories](#) - (polyclonal, peptides)
18. [BIOCON, Inc.](#) - (monoclonal, polyclonal) - *in vitro*
19. [Bioscience International](#) - (monoclonal, polyclonal)
20. [BioDiversa](#) - (monoclonal, polyclonal, peptides)
21. [Bio-Express](#) - (monoclonal) - *in vitro* and IgG fragment production
22. [Biogenes](#) - (monoclonal, polyclonal, peptides)
23. [Biogenesis](#) - (monoclonal, polyclonal, peptides)
24. [Bioinvent International AB](#) - (monoclonal) - human monoclonal antibodies
25. [NEW Bionexus](#) - (monoclonal, polyclonal)

EXHIBIT A

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26. [Bionostics, Inc.](#) - (**monoclonal, polyclonal**)
27. [Bioquest](#) - (**monoclonal, polyclonal**)
28. [BioSource International](#) - (**monoclonal, polyclonal**)
29. [Bio-Synthesis](#) - (**monoclonal, polyclonal, peptides**)
30. [Biotrend](#) - (**monoclonal, polyclonal, peptides**)
31. [Biovendor](#) - (**monoclonal, polyclonal**)
32. [Bioworld](#) - (**monoclonal, polyclonal, peptides**)
33. [**NEW** Cambridge Research Biochemicals](#) - (**polyclonal, peptides**)
34. [Capralogics](#) - (**polyclonal**)
35. [Cell Essentials](#) - (**monoclonal, polyclonal, peptides**) - bioreactor production and antibody purification
36. [**NEW** Cemines](#) - (**monoclonal, polyclonal**)
37. [Charles River Laboratories](#) - (**polyclonal**)
38. [Charles River Laboratories SPAFAS](#) - (**polyclonal**) - custom manufacturing of antibodies (antiserum or IgY) to mammalian antigens as well as human proteins. The unique approach utilizing Specific Pathogen Free (SPF) chickens produces high quality antibodies without interference or cross-reactivity due to the presence of antibodies to common poultry pathogens
39. [Cosmix](#) - phage-display based services including custom mouse Fab antibodies
40. [Covalab](#) - (**monoclonal, polyclonal, peptides**) - chicken antibodies
41. [Covance Research Products](#) - (**monoclonal, polyclonal**)
42. [Custom Monoclonals International](#) - (**monoclonal**)
43. [Diatec](#) - (**monoclonal**)
44. [Eurogentec](#) - (**monoclonal, polyclonal, peptides**) - chicken antibodies
45. [Fitzgerald Industries International, Inc.](#) - (**monoclonal, polyclonal**)
46. [Flock Antibodies](#) - (**polyclonal**) - chicken antibodies
47. [Gallina Biotechnology](#) - (**polyclonal**) - chicken antibodies
48. [Gallus Immunotech](#) - (**polyclonal**) - chicken antibodies
49. [Geneka Biotechnology, Inc.](#) - (**polyclonal**)
50. [Genemed Synthesis, Inc.](#) - (**monoclonal, polyclonal, peptides**)
51. [Genosys](#) - (**polyclonal, peptides**)
52. [**NEW** Genovac](#) - cDNA based immunization - (**monoclonal**)
53. [Gramsch Laboratories](#) - (**monoclonal, polyclonal, peptides**)
54. [Green Mountain Antibodies, Inc.](#) - (**monoclonal**)
55. [Harlan Bioproducts](#) - (**monoclonal, polyclonal, peptides**)
56. [**NEW** Harlan Sera-Lab Limited](#) - (**monoclonal, polyclonal, peptides**) - *in vitro* production, fragmentation, conjugation, cell banking, and MAP testing
57. [ICN Biomedicals, Inc.](#) - (**polyclonal**)
58. [Imgenex](#) - (**monoclonal, polyclonal**)
59. [Immunechem](#) - (**polyclonal**)
60. [Immunochem Diagnostics Technology, Inc.](#) - (**monoclonal, polyclonal**)
61. [Immunological Resource Center](#) - (**monoclonal, polyclonal**) - *in vitro* production
62. [Immunosystem](#) - (**polyclonal**) - chicken antibodies
63. [ISL \(Immune Systems Ltd\)](#) - (**monoclonal, polyclonal, peptides**)
64. [Lampire Biological Laboratories](#) - (**monoclonal, polyclonal**)
65. [Lee Laboratories](#) - (**polyclonal**)
66. [MorphoSys](#) - antibodies of fully human composition
67. [Maine Biotechnology Services, Inc.](#) - (**monoclonal, polyclonal, peptides**) - *in vitro* production, purification, fragmentation, and conjugation
68. [Mathison Immuno Scientific, Inc.](#) - (**monoclonal**)
69. [Mediclone](#) - (**monoclonal**)

70. [MicroPharm Ltd.](#) - (**polyclonal**)
71. [NEW Neoclone](#) - (**monoclonal**)- polyol-responsive nAbs™
72. [Panigen](#) - (**monoclonal, polyclonal**)
73. [NEW Pepscan Systems](#) - (**peptides**) - immunogen synthesis and epitope mapping
74. [Pocono](#) - (**monoclonal, polyclonal**)
75. [ProtoPROBE, Inc.](#) - (**monoclonal, polyclonal, peptides**) - recombinant single chain frag variables (ScFv) and recombinant phage antibodies
76. [ProSci, Inc.](#) - (**polyclonal, peptides**)
77. [QED Biosciences, Inc.](#) - (**monoclonal, polyclonal**) - *in vitro* production, anti-idiotypic and bifunctional antibody production as well
78. [Quality Bioresources, Inc.](#) - (**polyclonal**)
79. [Quality Controlled Biochemicals Corporation](#) - (**polyclonal, peptides**)
80. [Research Genetics](#) - (**polyclonal, peptides**)
81. [Rockland](#) - (**monoclonal, polyclonal, peptides**) - tissue culture mAb production and DN/ immunizations (with vector construction)
82. [Southern Biotechnology Associates, Inc.](#) - (**monoclonal, polyclonal, peptides**)
83. [Spring Valley Laboratories](#) - (**monoclonal, polyclonal, peptides**)
84. [NEW Strategic Biosolutions](#) - (**monoclonal, polyclonal**)
85. [Terra Nova Biotechnology](#) - (**monoclonal, polyclonal**) - *in vitro*
86. [NEW Virusys](#) - (**monoclonal, polyclonal, peptides**) - chicken antibodies and conjugation s
87. [Washington Biotechnology, Inc.](#) - (**monoclonal, polyclonal, peptides**)
88. [Yes Biotech Laboratories, Ltd.](#) - (**monoclonal**)
89. [Zymed](#) - (**monoclonal, polyclonal, peptides**)

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Alteration of Frizzled Expression in Renal Cell Carcinoma

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Key Words

β -Catenin · Cyclin D1 · Frizzled receptor · Renal cell carcinoma · Wnt

Abstract

To evaluate the involvement of frizzled receptors (Fzds) in oncogenesis, we investigated mRNA expression levels of several human Fzds in more than 30 different human tumor samples and their corresponding (matched) normal tissue samples, using real-time quantitative PCR. We observed that the mRNA level of Fzd5 was markedly increased in 8 of 11 renal carcinoma samples whilst Fzd8 mRNA was increased in 7 of 11 renal carcinoma samples. Western blot analysis of crude membrane fractions revealed that Fzd5 protein expression in the matched tumor/normal kidney samples correlated with the observed mRNA level. Wnt/ β -catenin signaling pathway activation was confirmed by the increased expression of a set of target genes. Using a kidney tumor tissue array, Fzd5 protein expression was investigated in a broader panel of kidney tumor samples. Fzd5 membrane staining was detected in 30% of clear cell carcinomas, and there was a strong correlation with nuclear cyclin D1 staining in the samples. Our data suggested that altered expression of certain members of the

Fzd family, and their downstream targets, could provide alternative mechanisms leading to activation of the Wnt signaling pathway in renal carcinogenesis. Fzd family members may have a role as a biomarker.

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Introduction

The Wnt signaling pathway is evolutionary conserved and controls many events during embryonic development. Members of the Wnt gene family of secreted glycoproteins are involved in embryonic induction, generation of cell polarity, cell proliferation and the determination of cell fate [1, 2]. Recently, it has become evident that the Wnt pathway is also deregulated in a range of tumors [3].

The Wnt signaling pathway is activated when Wnt proteins bind to a cell surface receptor complex consisting of a member of the frizzled receptor (Fzd) family and either low-density-lipoprotein receptor-related protein (LRP)5 or LRP6 [4, 5]. A detailed characterization of the Fzds and the immediate downstream events after Wnt binding has been hampered by the lack of pure biologically active Wnts.

Downstream of the receptor complex, three pathways may be initiated, depending on the composition of the

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ligand and receptor complex. The 'Wnt/ β -catenin pathway', the 'Wnt/ Ca^{2+} pathway' or the 'Wnt polarity pathway' [6]. The Wnt/ β -catenin pathway has been linked to carcinogenesis. Genetic alterations in components of this pathway (adenomatous polyposis coli, APC, axin and β -catenin) can result in the accumulation of non-phosphorylated β -catenin [3, 7] and this can promote carcinogenesis. Conversely, neither the Wnt/ Ca^{2+} pathway nor the Wnt polarity pathway involves the activation of β -catenin [for review, see ref. 1, 6].

Mutations in one of the three regulatory genes (APC, β -catenin and axin), overexpression of Wnts and Fzds or the expression of a constitutively active Fzd have been linked to Wnt/ β -catenin pathway activation in various tumors [8, 9].

To evaluate the involvement of Fzds in oncogenesis, we investigated mRNA expression levels of several human Fzds (Fzd2, 3, 5, 6, 7, 8 and 9) in more than 30 different human tumor samples using real-time quantitative PCR. Each sample was compared with its corresponding (matched) normal tissue sample. The most striking observation was the dramatically increased Fzd5 and Fzd8 mRNA expression seen in the renal carcinoma samples. This was confirmed at the protein level using Western blotting. Kidney tumor tissue arrays confirmed Fzd5 membrane staining in 30% of clear cell carcinomas, with nuclear cyclin D1 showing a strong correlation with the Fzd5 membrane labeling. Fzd8 protein expression analysis was not performed due to the lack of suitable reagents. These data suggest that Fzd5 may have a role in renal cell carcinogenesis due to its frequent overexpression observed in these tumor samples. Potential future applications could include uses in tumor targeting or as a potential biomarker.

Materials and Methods

Tissue Samples

Frozen tumor tissue samples with corresponding normal tissue from the same patient were derived either from human biopsy or autopsy material (Department of Pathology, University of Antwerp, kindly provided by Prof. E. Van Marck). Tissue specimens were snap-frozen in liquid nitrogen and kept at -80°C until use. Frozen sections of kidney tumor and normal tissue samples were stained with hematoxylin-eosin to support the pathologist's observations and to confirm the type of kidney tumor. Paraffin-embedded tissue slides of renal carcinoma, lung carcinoma, breast and colon carcinoma were obtained, after encryption, from the Department of Pathology (Middelheim Hospital, Antwerp, Belgium). The CL1 human kidney cancer (SuperBioChips Laboratories) tissue array used in this study contained 59 tissue samples consisting of 9 normal kidney tissues,

30 clear cell renal carcinoma samples and another 20 renal cell tumor types (chromophil, chromophobe, papillary type, collecting duct carcinoma and samples with mixed types).

RNA Isolation and Reverse Transcription

Total RNA was extracted from tissue specimens using Ultraspec Reagent (Biotecx, USA) according to the manufacturer's instructions. All total RNA was routinely treated with DNase (DNA-free kit, Ambion, USA). 1 μg of total RNA was used to synthesize cDNA using oligo-dT primers (Superscript; Invitrogen, Merelbeke, Belgium). Reverse transcription was performed at 42°C for 60 min, followed by 70°C for 10 min.

Real-Time PCR

Real-time PCR was performed on either an ABI Prism 7700 or 7900 Sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, Calif., USA) using the 5' nuclease assay (TaqmanTM). Primer and probe sequences were designed using Primer Express (PE Applied Biosystems) and are shown in table 1. Quantitative values were obtained from the threshold cycle number (Ct) at which the increase in the signal associated with exponential growth of PCR products is detected using PE Biosystems analysis software, according to the manufacturer's instructions.

We have used the $2^{-\Delta\Delta\text{Ct}}$ method to analyze the relative changes in gene expression of the different genes between tumor and corresponding normal tissue samples. We used the mitochondrial ATP synthase 6 (ATP6) as the endogenous RNA control [10; Janssens et al., in prep.], and each sample was normalized to its ATP6 content. The relative expression of the target gene was also normalized to the corresponding normal tissue sample (calibrator). Results, expressed as the amount of target sample relative to the ATP6 gene and the calibrator, were determined as follows, $N = 2^{-(\Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{calibrator}})}$, where the ΔCt values of the sample and calibrator were determined by subtracting the average Ct value of the sample and the calibrator from the average Ct value of the ATP6 gene. Amplification was done essentially as described previously [10]. Briefly, 50 μl of reaction mixture containing 1 μl of cDNA template were amplified as follows: incubation at 50°C for 2 min, denaturation at 95°C for 10 min, and 50 cycles at 95°C for 15 s and 60°C for 1 min.

Membrane Preparation, Gel Electrophoresis and Immunoblotting

Tissue samples were weighed, suspended at a 40 times dilution [= 40 volumes/original wet weight of tissue (v/w)] in 50 mM Tris-HCl buffer, pH 7.4, and homogenized with an Ultra-Turrax homogenizer. After centrifugation for 10 min, 24,000 g at 4°C , the pellet was washed three times by resuspension in the Tris-HCl buffer followed by centrifugation. The final membrane pellets were stored at -80°C in the Tris-HCl buffer at a concentration of 0.5–1 mg/ml. The Bradford protein assay (Pierce, Aalst, Belgium) was used for protein determination. Proteins (50 μg) were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. After primary and secondary antibody incubation, the antigen-antibody-peroxidase complex was detected by chemiluminescence (Pierce, Aalst, Belgium) according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry was performed on 10- μM -thick cryosections of unfixed tumor tissue and on 6- μM -thick paraffin sections from renal tumor tissue fixed by formalin or by an alcohol-based fixative. Adjacent tissue blocks from renal tumors were processed with

Table 1. Real-time PCR primer and probe sequences

Target cDNA	Primer/probe sequences ^a	Fragment position ^b	Accession No. ^c
FZD2	(a) 5'-atcccggtcccggc-3' (b) 5'-gtattgacatgtagaccgtgaagtc-3' (c) 5'-FAM-tacaccccgcatgtcgc-TAMRA-3'	1,548–1,613	AB017364
FZD3	(a) 5'-tcacgccagtgcatggg-3' (b) 5'-ttgtcacctcaattttattcatcg-3' (c) 5'-FAM-catccccggaacttaaccatcatcctttt-TAMRA-3'	1,473–1547	AB039723
FZD5	(a) 5'-tgccaaggtcacttcggtt-3' (b) 5'-tctccaagtcgcccgc-3' (c) 5'-FAM-ccttcatgggtgctgttgcccc-TAMRA-3'	2,143–2,204	HSU43318
FZD6	(a) 5'-ctagacccccaggttaagagaa-3' (b) 5'-cccagagagtcgtggagatggat-3' (c) 5'-FAM-tgtggtgaacctgcctgcgcag-TAMRA-3'	2,094–2,170	AF072873
FZD7	(a) 5'-cctgtggaaaggcataactgtg-3' (b) 5'-aaccaacgggaaacctcaga-3' (c) 5'-FAM-aagcaactttataggcaaaagcagcgcaa-TAMRA-3'	2,687–2,762	AB017365
FZD8	(a) 5'-tgtgtcggtgctctgctt-3' (b) 5'-cgctccatgtcgataaggaag-3' (c) 5'-FAM-ccaccttgcgcaccgtctcca-TAMRA-3'	853–919	AB043703
FZD9	(a) 5'-ccccgggagctacggac-3' (b) 5'-tagtcatgtgcaagaccacgg-3' (c) 5'-FAM-tggcacgcactgccactataaggct-TAMRA-3'	1,696–1,763	HSU82169
ATP5b	(a) 5'-gggttaggtgtgccttgggt-3' (b) 5'-gggcgcagtgattataggctt-3' (c) 5'-FAM-aagtggtgtagggcatttttaatttagagcg-TAMRA-3'	580–503	AF368271
c-myc	(a) 5'-accaccagcagcactctga-3' (b) 5'-tccagcagaaggatgccagact-3' (c) 5'-FAM-accttttgcaggagcctgcctct-TAMRA-3'	1,297–1,413	HSMYC1
Cyclin D1	(a) 5'-gaacctggccgcaatgac-3' (b) 5'-cgccctcggcattttgga-3' (c) 5'-FAM-ccgcacgatttcattgaacactt-TAMRA-3'	4,148–4,211	AF511593
PPAR δ	(a) 5'-agcatcctcacggcaaa-3' (b) 5'-gtctcgatgtcgtggatcaca-3' (c) 5'-FAM-ccagccacacggccct-TAMRA-3'	932–990	NM-006238

^a (a) = Sense primer; (b) = antisense primer; (c) = probe.
^b Fragment positions are given according to the EMBL/GenBank accession No. of cloned sequence.
^c EMBL/GenBank accession No. of cloned sequence.

formalin and with the alcohol-based fixative. Paraffin and cryosections were mounted on poly-L-lysine or 3-aminopropyltriethoxysilane-gelatin-coated slides. The 59 tissue samples on the CL1 human renal cancer tissue array slides were all fixed with formalin and embedded in paraffin, and the sections were mounted on silane-coated slides (SuperBioChips Laboratories). In addition to renal carcinoma tissue, sections from 10 formalin-fixed paraffin-embedded lung carcinomas were stained for Fzd5. Colon and breast tumors

were used as positive controls for β -catenin and cyclin D1 immunostaining.

The following primary antibodies were used: Fzd5 (Upstate Biotechnology), β -catenin (Zymed), cyclin D1 (Zymed), E-cadherin (Novocastra) and cytokeratin 8 (Biogenex). Cryosections were fixed in 4% paraformaldehyde for 5 min; acetone for 5 min at -20°C and 70% ethanol for 5 min. Endogenous peroxidase activity was quenched using 3% H_2O_2 . Paraffin sections of formalin- and alcohol-

Table 2. Fzd mRNA expression in tumor samples

Sample ^a	Tissue	Tumor type	x-fold expression increase ^b						
			FZD2	FZD3	FZD5	FZD6	FZD7	FZD8	FZD9
133702	kidney	adenocarcinoma	0.17	0.5	3.72	2.13	0.06	1.32	–
137770	kidney	renal cell carcinoma	1.23	3.56	8.26	2.61	1.3	8.21	5.44
138844	kidney	renal cell carcinoma	0.31	0.11	6.84	1.18	0.23	3.18	2.8
137146	kidney	renal cell carcinoma	0.47	0.45	3.16	2.23	0.73	4.42	1.37
137564	kidney	renal cell carcinoma	3.43	2.95	9.6	1.57	7.64	3.52	–
133408	kidney	renal cell carcinoma	23.97	0.98	6.39	2.48	5.05	16.72	–
139188	kidney	renal cell carcinoma	3.7	0.56	0.66	1.33	0.37	4.41	1.53
135699	kidney	renal cell carcinoma	2.6	0.36	4.83	0.9	0.34	2.54	2.31
139064	kidney	renal cell carcinoma	5.16	1.82	1.25	2.36	1.8	2.19	2.85
134585	kidney	renal cell carcinoma	1.47	0.72	1.38	0.47	0.09	0.6	–
140279	kidney	renal cell carcinoma	7.33	18.17	3.93	6.05	6.41	4.65	7.91
137252	ovary	carcinosarcoma	0.4	3.39	0.49	0.44	0.92	0.54	0.53
138256	ovary	papillary carcinoma	0.7	5.17	1.22	2.19	0.4	0.11	–
146472	ovary	serous papillary carcinoma	0.39	3.29	2.56	1.34	3.94	0.67	0.59
145845	colon	adenocarcinoma	3.45	3.36	0.58	1.22	1.67	1.24	1.99
146145	colon	adenocarcinoma	5.46	6.74	4.42	6.57	3.36	6.08	0.15
146630	colon	adenocarcinoma	4.01	4.73	0.3	1.16	0.54	0.55	0.12
146633	colon	adenocarcinoma	1.87	1.07	1.62	1.47	0.51	2.07	–
147055	colon	adenocarcinoma	0.66	1.41	1.01	1.33	0.24	0.79	4.87
142253	lung	adenocarcinoma	0.67	5.17	0.43	1.87	0.33	0.59	0.41
143036	lung	adenocarcinoma	0.67	1.24	2.63	1.13	1.11	1.04	9.19
138938	lung	adenocarcinoma	0.93	0.88	1.07	1.3	1.73	0.38	0.76
133563	lung	adenocarcinoma	2.76	1.23	0.31	1.34	0.99	0.78	4.97
144387	lung	adenocarcinoma	12.85	0.43	0.49	0.25	2.73	0.54	–
137304	lung	acinary adenocarcinoma	0.54	9.15	1.65	1.25	0.63	3.41	2.64
144546	lung	epithelial carcinoma	0.1	0.67	0.21	8.11	0.27	0.97	1.32
137621	lung	epithelial carcinoma	1.52	2.19	0.37	2.26	0.52	0.45	1.06
145552	lung	epithelial carcinoma	1.09	–	0.16	0.91	1.47	0.13	0.5
143987	testis	embryonal carcinoma	0.24	0.6	19.43	1.33	23.12	2.43	0.33
137332	stomach	leiomyoma	19.32	3.91	0.45	1.53	18.98	32.82	16.7
139026	stroma	gastrointestinal carcinoma	66.1	3.51	0.03	6.67	8.44	2.28	20.82
136049	rectum	adenocarcinoma	1.71	0.52	0.4	0.54	0.29	0.88	0.28
140794	gall bladder	adenosquamous carcinoma	0.19	–	0.93	3.02	0.2	–	–

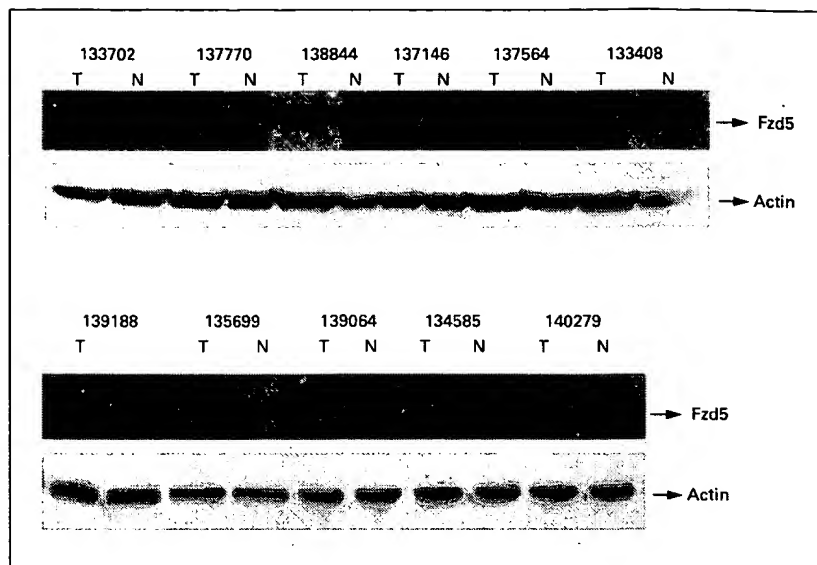
^a Sample identification numbers were given by the pathologist.

^b Results are expressed as x-fold increase of the gene in the tumor tissue sample compared to its matched normal tissue sample after normalizing both samples on the basis of their ATP5b content. A cutoff of 3-fold was used to define differential expression. Significant (>3-fold) increases in the expression level of the Fzd receptor are shown in italics. – = Expression of the target gene undetectable in one or both samples (tumor and/or normal).

fixed tissue were processed with a trypsin-citrate-microwave pretreatment or with an EDTA-microwave pretreatment to unmask epitopes, respectively. Sections were then sequentially processed with primary antibodies, biotinylated secondary antibodies and streptavidin-biotin-peroxidase (Fzd5, E-cadherin and cytokeratin 8). For β -catenin, polyclonal rabbit antibody with the EnVision detection system (DAKO) was used. The slides were further developed using 3-amino-9-ethylcarbazole, counterstained with hemalaun and mount-

ed with glycerin gelatin. Stained sections were observed with an Axioplan 2 microscope equipped with an Axiocam digital camera. Staining intensity for β -catenin was scored as no staining (value 0), weak and fragmentary staining of cell membranes (value 1), moderate membrane staining of less than 50% of the tumor cells (value 2), moderate membrane staining of more than 50% of tumor cells (value 3) and strong membrane staining of more than 75% of tumor cells (value 4). The cyclin D1 staining was quantified as a percentage of

Fig. 1. Fzd5 protein expression in matched tumor/normal kidney samples. T = Tumor sample; N = matched normal sample. Sample identification numbers are given by the pathologist.



cyclin D1-immunoreactive nuclei in tumor cells in three fields (area: 18,641 μm^2) of each tumor sample. The total number of tumor nuclei ranged from 51 to 164. The correlation between Fzd5 and β -catenin staining, and between Fzd5 and cyclin D1 staining was evaluated by the Mann-Whitney U test.

Results

Fzd mRNA Expression in Matched Human Tumor/Normal Tissue Samples

Fzd expression in tumor tissue was compared with Fzd expression in matched normal tissue samples and normalized to the expression of the housekeeping gene mitochondrial ATP5b (table 2). A 3-fold increase was considered significant.

In the kidney tumor samples, in which 10 of 11 samples were clear cell carcinomas, Fzd5 was upregulated in 8 of the 11 samples. A similar observation was made for Fzd8 and Fzd2, which were upregulated in 7 and 5 renal tumor samples, respectively. None of the other Fzds showed consistent upregulation.

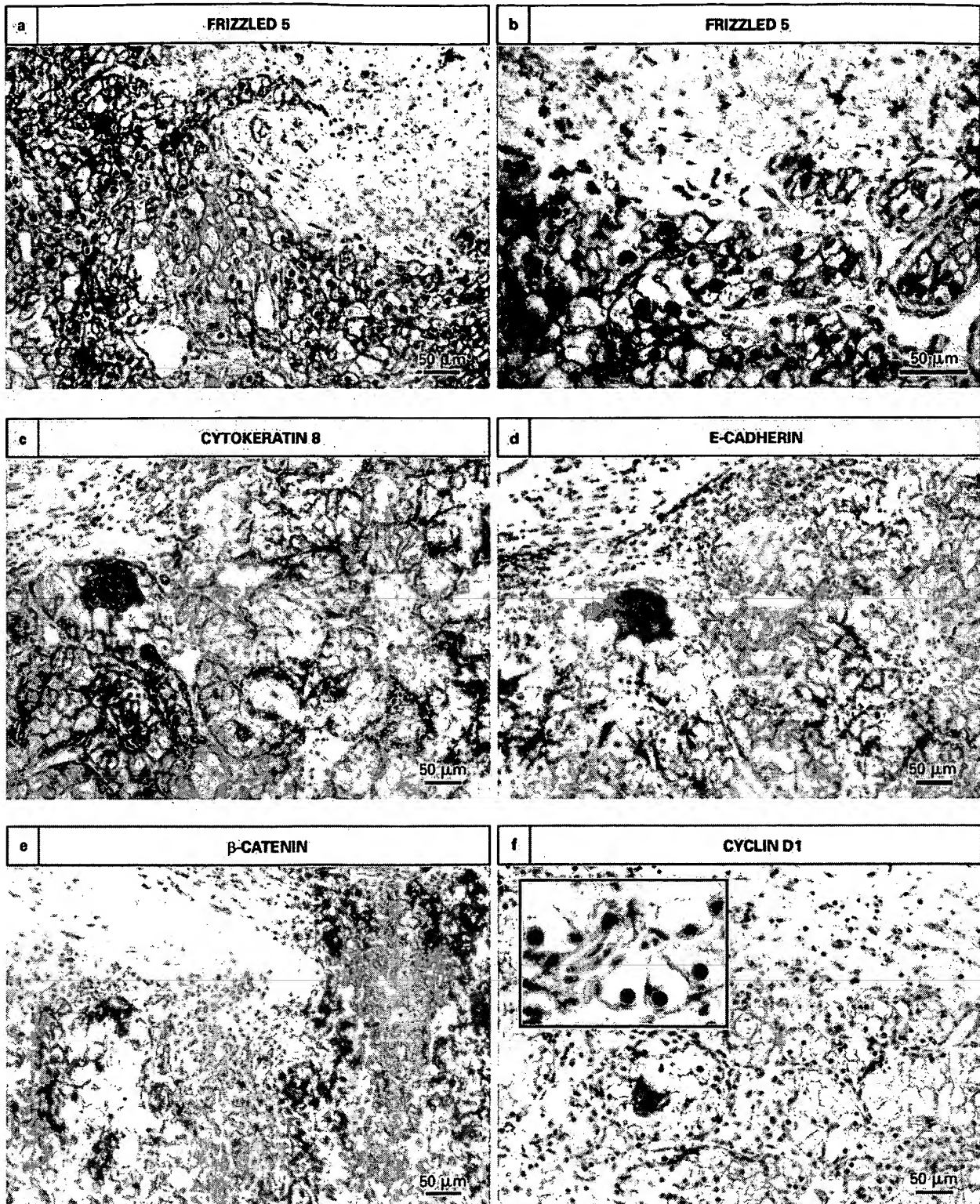
Both Fzd2 and Fzd3 were upregulated in 3 of 5 colon adenocarcinoma samples. No other Fzd expression was significantly different compared to the normal colon tissue sample. Fzd3 showed an increased expression in all 3 ovarian carcinoma samples. Fzd expression was not altered in any of the lung tumor samples. The Fzd expres-

sion level was observed to be relatively low in these lung tissues compared to the other tissues investigated.

Western Blot and Immunohistochemistry Analysis on Renal Carcinomas

Western blotting was used to evaluate Fzd5 protein expression in the renal tissue samples used for mRNA expression analysis. Membrane fractions of the renal carcinoma and corresponding normal tissue samples were prepared. As previously shown (table 2), Fzd5 mRNA upregulation was detected in 8 of the 11 matched tumor/normal samples. Increased expression of Fzd5 protein was seen in membrane fractions from 9 of 11 samples (fig. 1). In most cases, concomitant increases in Fzd5 mRNA and protein levels were observed.

Hematoxylin-eosin staining of the cryosectioned tumors confirmed the presence of clear cell carcinoma. Fzd5 immunostaining in clear cell carcinoma (fig. 2a, b) was observed to be localized to cell membranes and to nuclei. Cytokeratin 8 (fig. 2c) and E-cadherin (fig. 2d) were also detected. E-cadherin labeling of cell membranes in clear cell carcinoma was less intense and patchy compared to epithelial cells of normal renal tissue. β -Catenin staining was confined to the cell membrane. β -Catenin levels in the clear cell carcinoma membranes were highly variable. Nuclear β -catenin staining was not observed in any of the samples. Epithelial cells in normal renal tissue showed intense membrane staining and some cytoplasmic



staining. In addition, weak β -catenin staining of endothelial cells was observed. A high number of cyclin D1-immunoreactive nuclei was observed in clear cell carcinoma (fig. 2f).

On the CL1 human kidney cancer tissue array, 30% ($n = 9$) of the clear cell carcinoma tumor samples ($n = 30$) showed Fzd5 immunoreactivity (fig. 3a). Membrane-associated β -catenin staining was observed in 33% of the Fzd5-positive tumor samples and 57% of Fzd5-negative clear renal cell carcinoma samples (table 3; fig. 3c, d). Again, nuclear β -catenin staining was never observed. Statistical analysis did not reveal a difference in the expression of β -catenin between Fzd5-positive and Fzd5-negative tumor samples (fig. 4a).

Nuclear cyclin D1 was observed in 89% of the Fzd5-positive clear cell carcinoma samples (table 3; fig. 3e). Only 38% of the Fzd5-negative clear cell carcinoma samples contained nuclear cyclin D1. Statistical analysis showed a significantly higher cyclin D1 expression in Fzd5-positive compared to Fzd5-negative tumor samples (fig. 4b).

c-myc, Cyclin D1 and Peroxisome

Proliferator-Activated Receptor δ Expression in Renal Carcinomas

Wnt/ β -catenin pathway activation in the kidney tissue samples was investigated looking at the expression of a number of target genes, which have previously been shown to be upregulated when the pathway is active. Gene expression of *c-myc*, cyclin D1 and peroxisome proliferator-activated receptor δ (PPAR δ) was analyzed. Increased expression of both *c-myc* and cyclin D1 genes have been implicated in cell proliferation, and carcinogenesis, and they represent two of the more important and closely studied target genes of the Wnt signaling pathway.

Fig. 2. Distribution of Fzd5 (a, b), cytokeratin 8 (c), E-cadherin (d) and β -catenin (e) immunoreactivity in paraffin sections from a renal tumor processed by an alcohol fixative. From the same tumor, a formalin-fixed block was used for cyclin D1 immunostaining (f). Fzd5 immunostaining shows distinct immunoreactivity in cell membranes and in nuclei of clear cell renal carcinoma. Clear cells are immunoreactive for cytokeratin 8. β -Catenin and E-cadherin staining of membranes is rather weak, and not uniform, in clear cell renal carcinoma. Nuclear β -catenin immunoreactivity was not observed. In clear cell renal carcinoma, many nuclei showed cyclin D1 immunoreactivity. The inset in f shows a detailed view of the cyclin D1 labeling of nuclei in clear cell renal carcinoma.

Expression of PPAR δ was investigated because it represents a direct target of the β -catenin pathway with T cell factor binding sites in its promoter. Expression of *c-myc* was found to be upregulated in 7 of 11, whilst cyclin D1 was upregulated in 10 of 11 kidney tumor samples (table 4). PPAR δ was upregulated in 9 cases. All three selected target genes showed a marked upregulation in the majority of renal tumors, which suggested that the Wnt/ β -catenin pathway was activated in these samples.

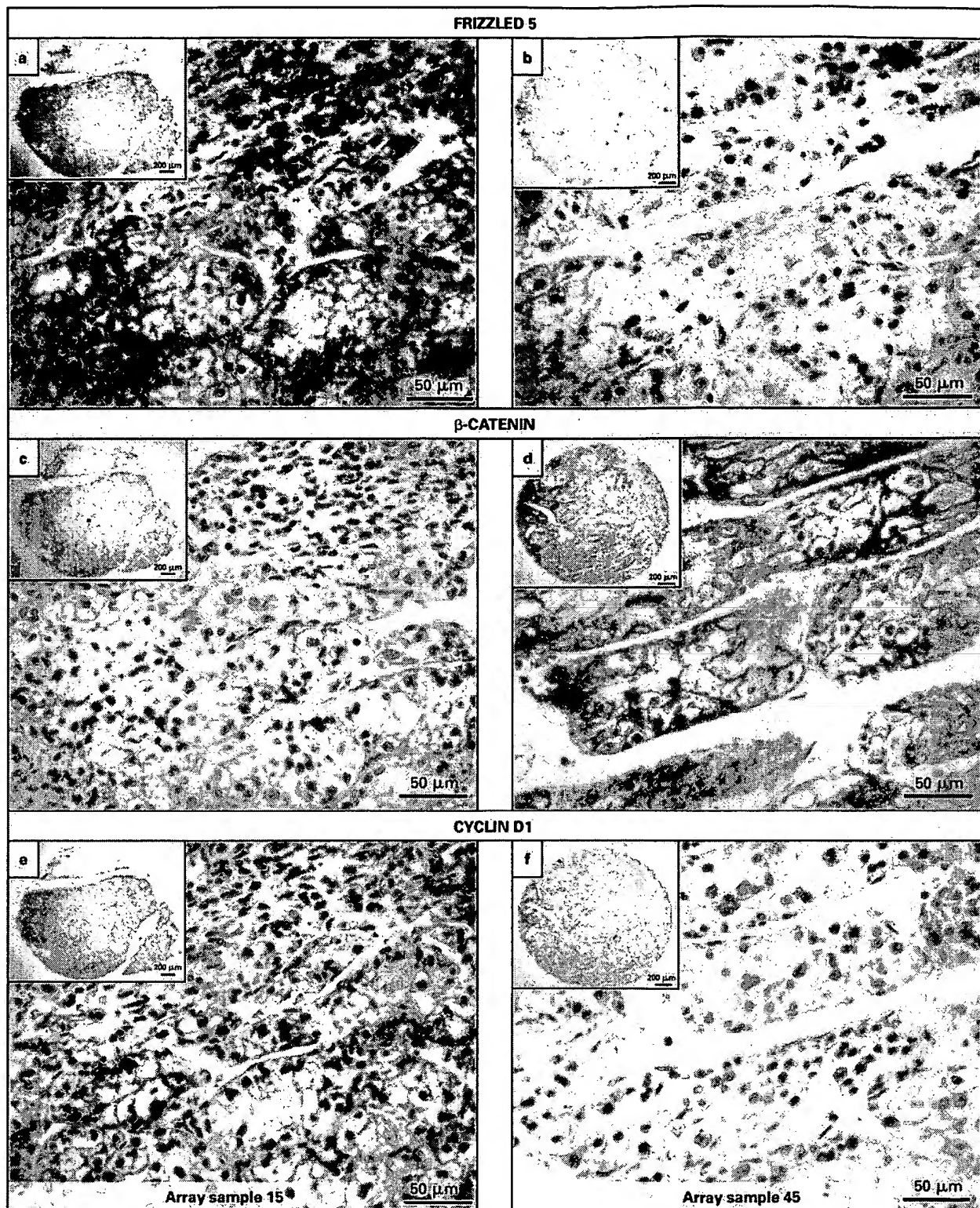
Discussion

Fzd family member overexpression has been postulated to play key roles in different tumor types such as esophageal carcinoma [11], gastric cancer [12] and head and neck squamous cell carcinoma [13]. The current study evaluated the potential implication of Fzds as tumor-associated antigens in different tumor types. We screened a number of matched normal/tumor tissue samples for the expression of a variety of Fzds using real-time quantitative PCR.

Results obtained revealed that both Fzd5 and Fzd8 mRNA were overexpressed in the majority of renal carcinoma samples when compared to the matched normal kidney samples. Fzd2 and Fzd3 were upregulated in 3 of 5 colon adenocarcinoma samples. Fzd3 was also upregulated in the ovarian tumor tissue samples compared to the matched normal tissue samples. None of the other Fzds evaluated showed a specific differential expression pattern in any of the samples studied. Fzd5 and Fzd8 show 69.1% similarity and belong to the same subgroup of Fzds [14]. The significantly higher expression of Fzd5 and Fzd8 in the renal tumor samples, as compared to the normal renal samples, suggests a higher probability that this subgroup may be implicated in the progression of renal cancer. Therefore, we decided to further examine the possible role of Fzd5 in renal carcinoma.

We observed, using Western blotting, that protein levels were mostly consistent with mRNA levels in the tumor samples. In order to be able to determine the Fzd5 expression in a broader range of kidney tissues, we utilized a tissue array. Fzd5 membrane staining was detected in 9 of 30 (30%) clear cell carcinomas, and importantly, membrane staining was not detected in the matched 9 normal kidney tissue samples.

Since the Wnt signaling pathway appears to play an important role in embryonic development, in particular embryonic kidney induction [15, 16], activation of this pathway in the adult kidney due to mutation or overex-



3

Table 3. Correlation between Fzd5 and β -catenin or cyclin D1 expression

	Fzd5, %	
	+	-
β -Catenin +	33	57
β -Catenin -	67	43
Cyclin D1 +	89	38
Cyclin D1 -	11	62

pression of one of the components of the pathway could be a determining factor in the development of renal cancers. Therefore, several studies have looked into the possible function the Wnt/ β -catenin pathway plays in renal carcinogenesis. APC gene mutations have been demonstrated not to be involved in renal carcinoma [17, 18]. In addition, β -catenin mutations are rare events in renal carcinoma [19, 20]. Nevertheless, cytoplasmic accumulation of β -catenin has been reported in a number of renal cell carcinomas [19], and thus the Wnt signaling pathway

Table 4. Wnt/ β -catenin target gene mRNA expression in tumor samples

Sample ^a	Tissue	Tumor type	x-fold expression increase ^b		
			c-myc	cyclin D1	PPAR δ
133702	kidney	adenocarcinoma	0.54	4.52	0.52
137770	kidney	renal cell carcinoma	13.9	28.91	5.53
138844	kidney	renal cell carcinoma	2.39	31.49	7.48
137146	kidney	renal cell carcinoma	7.62	15.38	3.15
137564	kidney	renal cell carcinoma	33.82	19.65	8.65
133408	kidney	renal cell carcinoma	7.8	8.92	4.86
139188	kidney	renal cell carcinoma	2.22	9.92	11.67
135699	kidney	renal cell carcinoma	12.18	22.73	5.53
139064	kidney	renal cell carcinoma	22.11	5.04	6.33
134585	kidney	renal cell carcinoma	1.79	1.14	1.67
140279	kidney	renal cell carcinoma	61.68	54.95	14.62

^a Sample identification numbers were given by the pathologist.

^b Results are expressed as x-fold increase of the gene in the tumor tissue sample compared to its matched normal tissue sample after normalizing both samples on the basis of their ATP56 content. A cutoff of 3-fold was used to define differential expression. Significant (>3-fold) increases in the expression level of the Fzd receptors are in italics.

Fig. 3. Fzd5, β -catenin and cyclin D1 immunostaining of the CL1 renal carcinoma tissue arrays. The left column of images represents serial sections from tumor sample 15. Insets show an overview of each tumor section on the serial tissue arrays. The Fzd5-immunoreactive clear cell renal carcinoma (a) of this tumor sample does not express β -catenin (c). Immunostaining for cyclin D1 (e) detects distinct labeling of nuclei in clear cell renal carcinoma. The right column of images is taken from serial sections of tumor sample 45. Clear cell renal carcinoma from this tumor sample does not express Fzd5 (b) and cyclin D1 (f) but does show distinct membrane β -catenin staining (d).

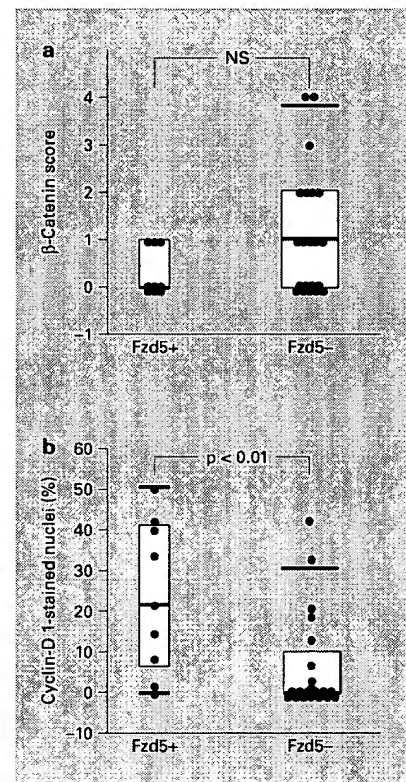


Fig. 4. Box plot charts (thick black line = median) illustrating the relationship between Fzd5 immunostaining and β -catenin (a) and cyclin D1 (b) expression in clear cell renal carcinoma. No significant correlation was observed between the β -catenin scores of Fzd5-positive and -negative clear cell renal carcinoma. Nuclear cyclin D1 staining in clear cell renal carcinoma showed a significant difference between Fzd5-positive and Fzd5-negative tumor samples.

might act as an inducer of tumorigenesis in the kidney. This view is supported by the observation that aberrant activity of the Wnt signaling pathway has been reported in renal-cancer-derived cell lines. Zang et al. [21] observed a higher expression level of Wnt5a and Fzd5 mRNA in the renal cancer cell line GRC-1 than in the normal renal cell line HK-2. Expression of β -catenin was also higher in GRC-1 than in HK-2.

To determine the status of the canonical Wnt signaling pathway in our renal carcinoma samples, we have quantitated the mRNA levels of three important target genes of T cell factor/lymphoid enhancer factor activation by β -catenin. The mRNA levels of these three target genes (c-myc, cyclin D1 and PPAR δ) correlated largely with the expression of Fzd5 in these samples, suggesting that the canonical pathway is activated. On the kidney tissue array, cyclin D1 protein expression showed a highly significant correlation with the Fzd5 expression in the tumor samples (table 3). Cyclin D1 protein is frequently overexpressed in various tumors, but in only a proportion of the cases is it due to amplification of the cyclin D1 gene [22]. Therefore, other mechanisms such as upregulation of gene transcription may play a substantial role in the overexpression of cyclin D1 [23–26]. Our data, showing increased cyclin D1 expression in renal carcinoma samples, are consistent with the results of Stassar et al. [27]. They studied genes that are associated with human renal carcinoma by suppression subtractive hybridization and reported 14 differentially expressed genes, including cyclin D1. Although we would have expected an increased nuclear β -catenin staining, nuclear accumulation of β -cate-

nin was not observed in any of the tumors or on the tissue array. This result is consistent with the data presented for renal cell carcinomas by Kim et al. [19]. They did not detect nuclear β -catenin staining in the 52 renal cell carcinomas examined. The lack of nuclear β -catenin staining has also been reported by others in tumors that might have arisen from Wnt/ β -catenin pathway activation [28–31].

While expression of both Wnt5a and Fzd5 does induce duplication of the *Xenopus* head, exogenous expression of Fzd5 in a *Xenopus* model does not induce duplication of the head [32]. Fzd5 does not activate the β -catenin signaling pathway on its own, as the presence of its endogenous ligand is also required. Our results suggest that Fzd5 may have a role in renal cell carcinogenesis due to its frequent overexpression observed in these tumor samples, and we hypothesize that if Fzd5 is overexpressed, it has a rather limited effect on β -catenin signaling. However, in the presence of its endogenous still unknown ligand, it activates the canonical Wnt signaling pathway. The elucidation of this ligand and its binding characteristics is still under investigation. Ultimately, knowledge of the specific expression patterns of both Wnt and Fzd members could lead to directed tumor targeting or could be used as a tumor marker.

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